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Evaluation of the Presence of 2-LTR HIV-1 Unintegrated DNA as a Simple Molecular Predictor of Disease Progression

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In a preliminary cross-sectional analysis of 109 human immunodeficiency virus type 1 (HIV-1)-infected subjects the presence of 2-long terminal repeat (LTR) unintegrated circular HIV-1 DNA in peripheral blood mononuclear cells (PBMC) was found to be associated with both symptomatic infection (P = 0.0037) and low CD4 counts (P = 0.0004). To investigate the prognostic significance of the presence of 2-LTR HIV-1 DNA, a subset of 23 2-LTR-negative and 25 2-LTR-positive asymptomatic individuals were followed up for 12–24 months. The two groups did not differ in terms of baseline CD4 counts, zidovudine (ZDV) therapy, and duration of HIV-1 infection. Longitudinal analysis of CD4 values did not indicate a significantly different CD4 outcome between the two groups. However, when only ZDV-treated subjects were considered, a significant (P = 0.042) decrease in CD4 counts was found at month 24 with respect to baseline in 2-LTR-positive (n = 12) but not in 2-LTR-negative (n = 11) patients. Moreover, when >40% CD4 loss from baseline and/or development of CDC stage B or C symptoms were considered as indicators of disease progression, there was a significantly higher number of events in the whole 2-LTR-positive group than in the whole 2-LTR-negative group (P = 0.0197 at month 12, P = 0.0299 at month 18, P = 0.0373 at month 24). Thus, the presence of 2-LTR HIV-1 DNA in PBMC merits further investigation as a simple, qualitative, molecular predictor of disease progression and decreased response to antiretroviral therapy. ***J. Med. Virol. 52:20–25, 1997.***

KEY WORDS: human immunodeficiency virus type 1; unintegrated DNA; disease progression; polymerase chain reaction; zidovudine

INTRODUCTION

Infection with human immunodeficiency virus type 1 (HIV-1) is usually followed by a prolonged asymptomatic period before development of the acquired immune deficiency syndrome (AIDS). A number of immunological, serological, and virological markers have been used as predictors of disease progression in HIV-1 infection [Tsoukas and Bernard, 1994]. More recently, advances in nucleic acid detection, particularly polymerase chain reaction (PCR) technology, have allowed the evaluation of several molecular virological parameters as novel direct markers of the course of HIV-1 infection. Indeed, quantitation of HIV-1 RNA in plasma [Bagnarelli et al., 1992; Piatak et al., 1993] and HIV-1 DNA and RNA [Bagnarelli et al., 1992; Gupta et al., 1993] in peripheral blood mononuclear cells (PBMCs) has indicated a significant association between viral load and disease stage. Other potentially useful molecular assays include relative quantitation of differently spliced HIV-1 transcripts [Furtado et al., 1995; Saksela et al., 1994], measurement of the ratio between integrated and unintegrated HIV-1 DNA [Dickover et al., 1992; Bush et al., 1993; Donovan et al., 1994], genotypic analysis for prediction of drug resistance [Larder et al., 1991], or enhanced pathogenicity [Pouchier et al., 1995] of viral strains. There is now a widespread consensus that molecular monitoring provides a direct assessment of HIV-1 infection and may play an essential role in the management of infected subjects. However, most protocols do not appear to be amenable to large-scale applications due to the need for fine tuning of cumbersome techniques, such as selective PCR [Larder et al., 1991; Pouchier et al., 1995] and quantitative amplifi-

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cation of nucleic acids [Bagnarelli et al., 1992; Gupta et al., 1993; Piatak et al., 1993]. In this context, the novel serological markers recently reported, i.e., tumor necrosis factor-alpha (TNF-alpha) and soluble TNF-alpha receptor [Aukrust et al., 1994; Bilello et al., 1996] as well as 90K tumor-associated antigen [Iacobelli et al., 1995] serum levels, could prove to be more practical, although indirect, predictors of disease progression.

The aim of the study was to evaluate whether a simple qualitative molecular parameter, namely, the presence of 2-long terminal repeat (LTR) unintegrated HIV-1 DNA in PBMCs, could be useful as a direct predictor of disease progression. Of the three retroviral unintegrated DNA forms (linear DNA, 1-LTR circular DNA, and 2-LTR circular DNA), the 2-LTR circular DNA uniquely contains two contiguous copies of the LTR region and is thus distinguishable from the other unintegrated forms as well as from the integrated provirus by PCR amplification of the LTR-LTR junction sequence [Jurriaans et al., 1992; Pauza et al., 1994]. Recent evidence suggests that 2-LTR extrachromosomal HIV-1 DNA has a short half-life in in vitro infection of PBMCs [Pauza et al., 1994]. In light of the possibility that 2-LTR unintegrated HIV-1 DNA is detected preferentially in newly infected cells in vivo, we tested PBMC samples from 109 infected patients and followed 48 of these for up to 24 months.

MATERIALS AND METHODS

Patients and Samples

Patients studied were staged according to the 1993 Centers for Disease Control (CDC) classification system [Centers for Disease Control and Prevention, 1992]. At the time of testing for the presence of 2-LTR unintegrated HIV-1 DNA, 31, 29, 10, 5, 8, 12, 2, and 12 subjects were in CDC stages A1, A2, A3, B1, B2, B3, C2, and C3, respectively. Twelve patients were under zidovudine (ZDV) monotherapy at the time of testing, while another 13 patients were started on ZDV monotherapy during the follow-up period. The remaining 23 subjects did not receive any antiretroviral therapy due to elevated CD4 counts or lack of consent. Forty-two uninfected subjects were analyzed as controls. PBMC DNA from the HIV-1-seropositive subjects and the uninfected controls was prepared by sodium dodecyl sulfate-proteinase K lysis, phenol-chloroform extraction, and ethanol precipitation as previously described [Zazzi et al., 1993a] and quantitated by spectrophotometry. PCR quality was confirmed by a 24-cycle amplification of the GH20/PC04 human beta-globin region [Saiki et al., 1988].

Amplification of the LTR-LTR Junction Sequence

One microgram of purified DNA and an HIV-1 LTR-LTR junction-specific set of nested primers were used in a highly sensitive two-step PCR protocol [Zazzi et al., 1993b]. Outer primers were LR29 (5′-CTGCTTAAGCCTCAATAAAGC-3′, position 514–534 in the HIV-1_{SF2} genome, GenBank accession number KO2007) and LR30 (5′-CTAGCTTGTAGCACCATCCA-3′, position 129–148). Inner primers were LR31 (5′-CTTGCCTTGAGTGCTTCAAG-3′, position 534–553) and LR32 (5′-TGCCAATCAGGGAAGTAGCC-3′, position 67–86). The annealing temperature was 56°C in both outer (25 cycles) and inner (30 cycles) PCR, and one-fiftieth of the first PCR product was used as the template for the second PCR. Amplification products were electrophoresed through 2.4% NuSieve/0.6% Seakem (FMC, Rockland, ME) agarose gels and stained with ethidium bromide. The expected length of the amplification product for a correct LTR-LTR junction in 2-LTR unintegrated circular HIV-1 DNA is 187 base pairs (bp).

Data Analysis

The difference in mean CD4 counts between the 2-LTR-positive and 2-LTR-negative subjects in the cross-sectional study was analyzed by Student's *t* test. Chi-square analysis was used to compare the frequencies of symptomatic infection and low CD4 counts in the 2-LTR-positive and 2-LTR-negative groups.

Follow-up data (CDC stage and CD4 count) were obtained at months 12, 18, and 24 for 48, 42, and 42 subjects, respectively, who were asymptomatic (CDC A) at entry. Two-way repeated measures analysis of variance was used to check differences in the degree of change in CD4 counts over time between the 2-LTR-positive and the 2-LTR-negative groups. Temporal changes in CD4 counts were also evaluated in each group separately by paired *t* test at different time points with respect to baseline. Student's *t* test was used to compare the two groups for the number of months elapsed since acquisition of HIV-1 infection (estimated at the midpoint between the last negative and the first positive test for HIV-1 antibody) and for CD4 counts at each time point. In addition, differences in the rate of disease progression in the 2-LTR-positive and 2-LTR-negative groups were measured by chi-square analysis at each time point. Disease progression was defined as the appearance of symptoms (shift from CDC A to B or C) and/or loss of >40% CD4 cells with respect to baseline. Chi-square or Fisher's exact test was used to compare the frequencies of ZDV treatment and of subject enrollment shortly (<4 months) after documented seroconversion in the two groups. Statistical procedures were performed with SigmaStat software (Jandel Scientific, Erkrath, Germany).

RESULTS

The LTR-LTR junction nested PCR procedure did not generate any ethidium bromide-visible product when PBMC samples obtained from 42 uninfected control subjects were analyzed. By contrast, 53 of the 109 (48.06%) HIV-1-infected individuals were positive for the presence of 2-LTR circles. In four cases, different PCR products were generated in addition to the predicted 187-bp product. DNA fragments of different sizes were the only PCR products in another three asymptomatic and 10 symptomatic subjects (11.93%). Although the heterogeneous irregular products were HIV-1-specific (data not shown), these 13 subjects were

TABLE I. Detection of 2-LTR Circular Unintegrated HIV-1 DNA in Infected Subjects: Distribution of Results With Respect to CDC Symptom Class and CD4 Numbers

		CDC symptom class			CD4/mm^3		
2-LTR DNA	Total	A	B	C	<200	200–499	≥500
Positive	53	30	15	8	22	20	11
Negative	43	37	5	1	5	13	25
Total	96	67	20	9	27	33	36

not included in subsequent data analysis. As shown in Table I, the prevalence of the correct LTR-LTR junction DNA was significantly higher in symptomatic (CDC B and C) compared to asymptomatic (CDC A) subjects ($P = 0.0037$) and in patients with <500 CD4/mm^3 compared to patients with ⩾500 CD4/mm^3 ($P = 0.0004$). Accordingly, mean CD4 counts were significantly different between 2-LTR-positive and 2-LTR-negative subjects (327 ± 261 vs. 537 ± 300, $P = 0.0008$).

To investigate the prognostic value of PCR-detectable 2-LTR unintegrated HIV-1 DNA in PBMCs, we next analyzed follow-up data (CDC class and CD4 counts) obtained at months 12, 18, and 24 for 42–48 initially asymptomatic subjects (Table II). Subjects included in the 2-LTR-positive (n = 25) and 2-LTR-negative (n = 23) groups were not different in terms of baseline CD4 counts (mean ± standard deviation, 398 ± 217 and 461 ± 269, respectively), ZDV therapy (13/25 and 10/23 subjects, respectively, treated within month 12), and time elapsed since documented seroconversion (mean ± standard deviation, 40 ± 30 and 35 ± 29 months, respectively). Subjects analyzed at <4 months after estimated seroconversion (patients 15, 16, 20, 21, 34, 36, 37, and 41) were present equally in both groups. When all CD4 values were subjected to quantitative statistical analysis, there was no significant difference in the degree of change over time between the 2-LTR-negative and the 2-LTR-positive groups. Also, mean CD4 counts neither changed significantly within each group nor were different between the two groups at any time point. However, when the population was divided on the basis of ZDV treatment, there was a significant ($P = 0.042$) decrease in mean CD4 counts at month 24 with respect to baseline in the 2-LTR-positive (n = 12), but not in the 2-LTR-negative (n = 11), ZDV-treated group, whereas differences in untreated subjects were not significant in any group (Fig. 1).

The two groups were next analyzed for the numbers of disease progression events (development of CDC B/C symptoms and/or loss of >40% CD4 cells with respect to baseline). A significantly higher rate of progression was observed in the whole 2-LTR-positive group compared with the whole 2-LTR-negative group within month 12 (8/25 vs. 1/23, $P = 0.0238$), 18 (11/23 vs. 2/22, $P = 0.0074$), and 24 (13/22 vs. 3/22, $P = 0.0040$). The difference remained significant at all time points when the nine subjects with <200 CD4/μl at baseline (meeting the present definition of AIDS, though asymptomatic) were not included in progression analysis. Also, the association between presence of 2-LTR circles and subsequent disease progression was significant within 18 ($P = 0.0093$) and 24 ($P = 0.0096$) months or within 24 months ($P = 0.0296$), when CD4 loss or development of symptoms, respectively, were separately considered as the endpoint. By contrast, the difference in the rate of progression between the asymptomatic subjects with <400 CD4/mm^3 (n = 25) and those with ⩾400 CD4/mm^3 (n = 23) at baseline was not significant at any time point (progressors were 3/25 compared with 6/23, 6/24 compared with 7/21, and 8/23 compared with 8/21 within months 12, 18, and 24, respectively). Accordingly, baseline mean CD4 counts were not different between progressors and nonprogressors (446 ± 298 and 416 ± 209, respectively). Also, progressors and nonprogressors did not differ significantly in terms of ZDV therapy (10/16 and 12/28 subjects, respectively, treated within month 12; nonprogressors with follow-up <24 months not included) and duration of HIV-1 infection (mean ± standard deviation, 36 ± 31 and 33 ± 24 months, respectively). Thus, the presence of 2-LTR circles at baseline was a significant predictor of disease progression in the asymptomatic population studied, whereas CD4 counts failed to provide prognostic information (Fig. 2).

DISCUSSION

While the presence of 2-LTR circular unintegrated HIV-1 DNA in vivo was first documented in brain tissues from AIDS dementia patients [Pang et al., 1990], only three studies [Jurriaans et al., 1992; Pauza et al., 1994; Nicholson et al., 1996] have detected 2-LTR HIV-1 DNA in PBMC samples from infected subjects. Data from our and previous studies are highly consistent, reporting detection of 2-LTR HIV-1 DNA in 60–80% of infected individuals and generation of HIV-1-specific PCR products of unexpected size in 15–30% of cases, possibly due to aberrant LTR-LTR junctions [Jurriaans et al., 1992] and/or PCR artifacts [Pauza et al., 1994]. The cross-sectional analysis by Pauza et al. [1994] also showed that 2-LTR DNA levels were inversely correlated with CD4 counts in untreated subjects but not in patients receiving antiretroviral therapy. Nicholson et al. [1996] reported recently that the presence of circular (1-LTR plus 2-LTR) unintegrated HIV-1 DNA was associated with symptomatic infection and ZDV therapy. The amount of 1-LTR HIV-1 DNA in PBMCs has also been investigated and was suggested to be correlated with HIV-1 p24 antigenemia and decrease in CD4 count [Jurriaans et al., 1995]. The present report is the first follow-up study investigating specifically the prognostic significance of the presence of 2-LTR circles in PBMCs.

TABLE II. Follow-Up of 2-LTR DNA-Positive and 2-LTR DNA-Negative HIV-1-Infected Asymptomatic Subjects

		Baseline		Month 12		Month 18		Month 24		
Patient	2-LTR DNA	CD4[a]	CDC[b]	CD4	CDC	CD4	CDC	CD4	CDC	ZDV[c] outcome
1	Negative	67	A3	48	A3	66	A3	44	A3	−42 Nonprogressor
2	Negative	162	A3	395	A3	305	A3	341	A3	2 Nonprogressor
3	Negative	192	A3	282	A3	140	A3	206	A3	23 Nonprogressor
4	Negative	197	A3	273	A3	288	A3	305	A3	7 Nonprogressor
5	Negative	230	A2	357	A2	303	A2	286	A2	−3 Nonprogressor
6	Negative	291	A2	180	A3	222	A3	440	A3	−9 Nonprogressor
7	Negative	307	A2	364	A2	227	B2	390	B2	−31 Progressor at month 18
8	Negative	312	A2	588	A2	405	A2	324	A2	— Nonprogressor
9	Negative	336	A2	384	A2	369	A2	520	A2	— Nonprogressor
10	Negative	377	A2	286	A2	403	A2	290	A2	−6 Nonprogressor
11	Negative	392	A2	301	A2	479	A2	384	A2	−24 Nonprogressor
12	Negative	400	A2	317	A2	282	A2	317	A2	11 Nonprogressor
13	Negative	414	A2	547	A2	432	A2	306	A2	−2 Nonprogressor
14	Negative	518	A1	561	A1	544	A1	382	A2	— Nonprogressor
15	Negative	528	A1	792	A1	483	A2	390	A2	— Nonprogressor
16	Negative	532	A1	492	A2	557	A2	378	A2	— Nonprogressor
17	Negative	551	A1	722	A1	700	A1	756	A1	— Nonprogressor
18	Negative	570	A1	897	A1	625	A1	725	A1	— Nonprogressor
19	Negative	610	A1	610	A1	NA[d]	NA	NA	NA	— Nonprogressor till month 12
20	Negative	661	A1	667	A1	810	A1	870	A1	— Nonprogressor
21	Negative	683	A1	552	A1	583	A1	334	A2	— Progressor at month 24
22	Negative	1,089	A1	612	A1	475	B2	937	B2	— Progressor at month 12
23	Negative	1,174	A1	1,444	A1	1,223	A1	923	A1	— Nonprogressor
24	Positive	23	A3	45	A3	10	B3	7	C3	1 Progressor at month 18
25	Positive	94	A3	82	A3	52	A3	108	B3	−2 Progressor at month 18
26	Positive	119	A3	265	A3	277	A3	NA	NA	1 Nonprogressor till month 18
27	Positive	138	A3	89	A3	112	A3	33	B3	−29 Progressor at month 24
28	Positive	184	A3	222	A3	241	A3	224	A3	3 Nonprogressor
29	Positive	238	A2	186	A3	NA	NA	100	A3	1 Progressor at month 24
30	Positive	252	A2	225	B2	330	B2	253	B2	−10 Progressor at month 12
31	Positive	294	A2	96	B3	144	B3	163	B3	3 Progressor at month 12
32	Positive	294	A2	328	A2	NA	NA	NA	NA	— Nonprogressor till month 12
33	Positive	364	A2	359	A2	288	A2	285	A2	−35 Nonprogressor
34	Positive	378	A2	567	A2	527	A2	518	A2	— Nonprogressor
35	Positive	396	A2	222	A2	174	A3	146	A3	5 Progressor at month 12
36	Positive	398	A2	371	A2	392	A2	414	A2	— Nonprogressor
37	Positive	399	A2	619	A2	622	A2	684	A2	— Nonprogressor
38	Positive	400	A2	501	A2	409	A2	513	A2	−2 Nonprogressor
39	Positive	400	A2	263	B2	NA	NA	252	B2	5 Progressor at month 12
40	Positive	468	A2	186	B3	352	B3	373	B3	13 Progressor at month 12
42	Positive	483	A2	260	A2	320	A2	NA	NA	8 Progressor at month 12
41	Positive	486	A2	882	A2	800	A2	726	A2	— Nonprogressor
43	Positive	520	A1	426	A2	475	A2	480	A2	— Nonprogressor
44	Positive	603	A1	718	A1	567	A1	550	A1	— Nonprogressor
45	Positive	620	A1	360	A2	320	A2	248	A2	— Progressor at month 12
46	Positive	729	A1	962	A1	730	B1	830	B1	— Progressor at month 18
47	Positive	761	A1	717	A1	NA	NA	NA	NA	— Nonprogressor till month 12
48	Positive	916	A1	529	A1	NA	NA	NA	NA	— Progressor at month 12

[a]CD4, CD4 cells/mm^3.
[b]CDC, disease stage according to the 1993 Centers for Disease Control classification system.
[c]ZDV, zidovudine therapy (numbers indicate the month of initiation with respect to baseline;—indicates no ZDV therapy).
[d]NA, not available.

Results obtained in our cross-sectional analysis confirm the association between 2-LTR circles and advanced HIV-1 disease in a population larger than those described in previous studies [Pauza et al., 1994; Nicholson et al., 1996]. Although the changes in mean CD4 counts over time were not shown to differ significantly between the 2-LTR-positive and the 2-LTR-negative groups, there was a significantly higher rate of disease progression in the 2-LTR-positive compared to the 2-LTR-negative population. This apparent discrepancy may be explained at least partially by the observation that in the 2-LTR-positive group the loss of CD4 cells at most time points in the progressors was compensated for by increasing CD4 counts in several nonprogressors, possibly due to initiation of ZDV therapy (patients 26, 28) or rebound CD4 rising after initial drop following seroconversion (patients 34, 37, 41). It is also worth emphasizing that mean CD4 counts did decline significantly in 2-LTR-positive but not in 2-LTR-negative subjects when the ZDV-treated subpopulation was considered. This finding is consistent with the reported decrease in unintegrated HIV-1 DNA following initiation of antiretroviral treatment [Dickover et al., 1992; Donovan et al., 1994]. Although the

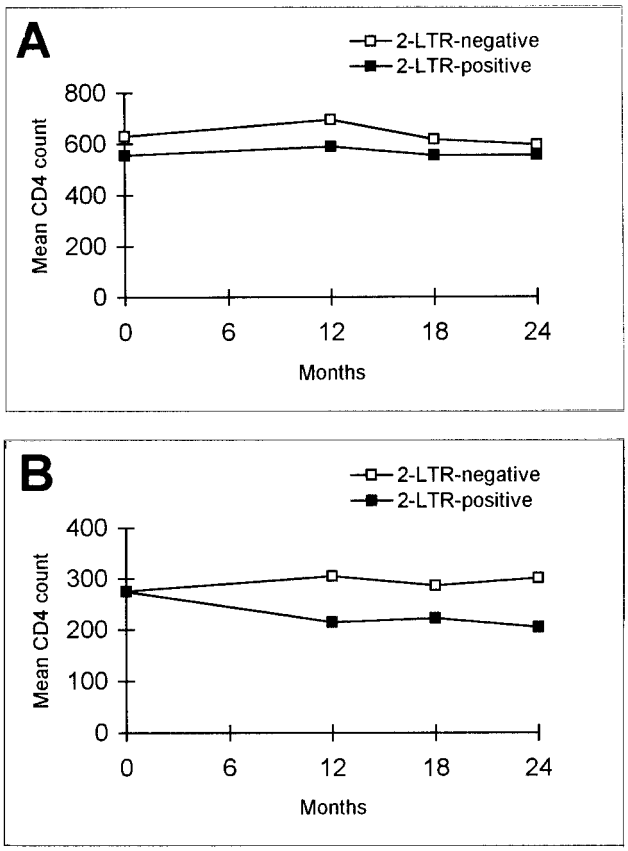

Fig. 1. Time course of mean CD4 counts in ZDV-untreated **(A)** and ZDV-treated **(B)** 2-LTR-negative and 2-LTR-positive subjects.

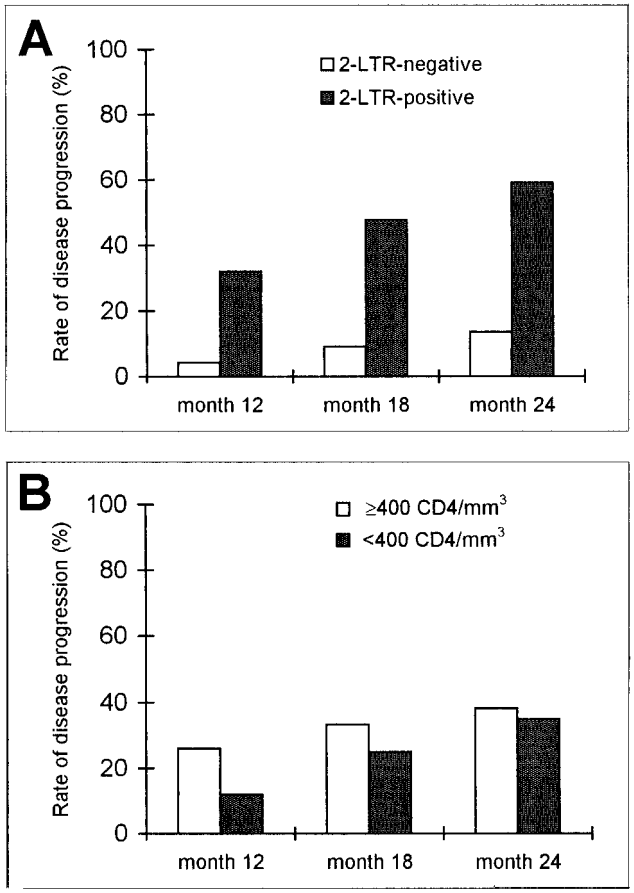

Fig. 2. Rate of disease progression in asymptomatic subjects with respect to presence of 2-LTR HIV-1 DNA **(A)** and to CD4 count **(B)** at baseline.

present report clearly supports the predictive value of the presence of 2-LTR HIV-1 DNA, further follow-up studies of larger populations are advisable in order to define to what extent detection of 2-LTR circles in PBMC is indicative of subsequent disease progression and decreased effectiveness of antiretroviral therapy.

The major prognostic indication of this study is that 2-LTR-negative asymptomatic subjects have a reduced risk of disease progression within 12–24 months. It is worth noting that another four 2-LTR-negative subjects (including the progressors 7 and 21) were 2-LTR-positive when 5 μg of PBMC DNA was used as the template (data not shown). Thus, the difference between 2-LTR-positive and -negative individuals may be quantitative rather than qualitative. High amounts of plasma and cellular HIV-1 RNA [Gupta et al., 1993; Katzenstein et al., 1996; Mellors et al., 1995; Merigan et al., 1996] and total HIV-1 DNA [Chevret et al., 1994; Gupta et al., 1993; Lee et al., 1994] have been similarly shown to be predictive of disease progression. 2-LTR unintegrated DNA could represent a relatively constant low proportion of total HIV-1 DNA, being detectable only in samples containing sufficiently high levels of viral DNA. In this context, depending on the sensitivity of the detection method, different amounts of PBMC DNA should be assayed in the first place in order to achieve optimal discrimination between 2-LTR-positive and -negative samples. Also, different DNA preparation procedures may affect variably relative recovery of integrated and unintegrated HIV-1 DNA, resulting in different changes in the sensitivity threshold of the detection method. Careful investigation of technical questions should precede the use of 2-LTR HIV-1 DNA as a prognostic marker of disease progression. Availability of a simple qualitative predictor of disease progression, as opposed to cumbersome and expensive quantitation of HIV-1 RNA or DNA, should increase the possibility for direct molecular monitoring of HIV-1 infection.

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